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Quantitative Detection of *Clostridium perfringens* in the Broiler Fowl Gastrointestinal Tract by Real-Time PCR

Mark G. Wise and Gregory R. Siragusa*

Poultry Microbiological Safety Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Russell Research Center, Athens, Georgia

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Strains of *Clostridium perfringens* are a frequent cause of food-borne disease and gas gangrene and are also associated with necrotic enteritis in chickens. To detect and quantify the levels of *C. perfringens* in the chicken gastrointestinal tract, a quantitative real-time PCR assay utilizing a fluorogenic, hydrolysis-type probe was developed and utilized to assay material retrieved from the broiler chicken cecum and ileum. Primers and probe were selected following an alignment of 16S rDNA sequences from members of cluster I of the genus *Clostridium*, and proved to be specific for *C. perfringens*. The assay could detect approximately 50 fg of *C. perfringens* genomic DNA and approximately 20 cells in pure culture. Measurements of the analytical sensitivity determined with spiked intestinal contents indicated that the consistent limit of detection with ileal samples was approximately 10^2 CFU/g of ileal material, but only about 10^4 CFU/g of cecal samples. The decreased sensitivity with the cecal samples was due to the presence of an unidentified chemical PCR inhibitor(s) in the cecal DNA purifications. The assay was utilized to rapidly detect and quantify *C. perfringens* levels in the gut tract of broiler chickens reared without supplementary growth-promoting antibiotics that manifested symptoms of necrotic enteritis. The results illustrated that quantitative real-time PCR correlates well with quantification via standard plate counts in samples taken from the ileal region of the gastrointestinal tract.

Clostridium perfringens is a gram-positive, anaerobic, rod-shaped, spore-forming bacterium that is capable of causing a broad spectrum of diseases in both humans and animals (19, 33). In broiler chickens, *C. perfringens* is associated with necrotic enteritis (NE), as excessive growth of the organism in the intestinal tract can lead to toxin production, which, in turn, can result in gut lesions (31). If untreated, the disease leads to an increase in bird mortality and/or liver condemnation at slaughter (16). Infection by parasites of the genus *Eimeria*, the use of protein-rich feed, and the feeding of small grains, such as wheat, rye and barley, predisposes birds to the risk of NE (1, 2, 15, 20, 21, 28, 32). The disease is routinely controlled by the prophylactic supplementation of feed or water with a variety of antibacterial drugs. As a consequence of concerns about the rise and spread of antibiotic-resistant bacterial strains, prophylactic and growth-promoting antibiotic treatment has recently come under greater scrutiny, and in some cases, it has been curtailed entirely (8). Unfortunately, with the withdrawal of antibiotic growth promoters, NE is expected to become more widespread (8). This phenomenon has already been observed in Western Europe, where restrictions and limitations on the use of growth-promoting and prophylactic antibiotics in chicken feed have lead to an increase in the prevalence of NE (11, 28).

At present, *C. perfringens* can be enumerated from chicken intestinal contents and feces by standard plate count methodologies. These are laborious and time-consuming procedures, as presumptive positive colonies have to be confirmed with further biochemical tests. Conventional PCR assays have been developed to rapidly detect *C. perfringens* in environmental samples (22, 23, 38). Real-time PCR offers the high sensitivity afforded by conventional PCR, but with the advantage that a post-PCR processing step is avoided, which allows for a savings in time and material. Additionally, real-time PCR can be quantitative over a much wider range, typically 5 to 6 $\log_{10}$, as opposed to conventional PCR in which the end-point DNA concentration is typically linear over only 2 to 3 $\log_{10}$ (10). Here, we report the development of a quantitative real-time PCR assay utilizing a fluorogenic, hydrolysis-type (5′ nuclease) probe to detect and quantify 16S rDNA sequences unique to *C. perfringens* retrieved from broiler chicken gastrointestinal contents. The assay is intended to be a quick and simple procedure that can supplant the need for direct plate counts in research endeavors that call for quantification of *C. perfringens*.

MATERIALS AND METHODS

Primer and probe design. The 16S rRNA gene sequences from members of cluster I of the genus *Clostridium* (12) were aligned using the ClustalW method available as part of the MegAlign program in the LaserGene sequence analysis package, version 5 (DNASTAR, Inc., Madison, WI). Regions unique to *C. perfringens* were identified, and putative oligonucleotide primers and probes were selected using the Primer3 program (34), by following the suggestions for fluorogenic, hydrolysis-type (5′ nuclease or TaqMan) primer/probe design (Applied Biosystems, Inc., Foster City, CA). After preliminary sensitivity and specificity testing with a number of candidate primer/probe sets, which included checking for potential cross-reactivity with the BLAST database search application (http://www.ncbi.nlm.nih.gov/BLAST) (3) and with the PROBE_MATCH program at the Ribosomal Database Project II (http://rdp8.cme.msu.edu/) (27), the following set was selected for further examination: forward primer CPerf165F (5′-CGCATAACGTTGAAAGATGG-3′), corresponding to *Escherichia coli* 16S rDNA positions 176 to 195, and reverse primer CPerf269R (5′-CCTTGGTAGGCCGTTACCC-3′), corresponding to *E. coli* positions 258 to 276. The probe, CPerf187F, corresponding to *E. coli* positions 194 to 219, was dual labeled with the dyes 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine

* Corresponding author. Mailing address: ARS-USDA, Russell Research Center, 950 College Station Road, P.O. Box 5677, Athens, GA 30605. Phone: (706) 546-3596. Fax: (706) 546-3772. E-mail: siragusa@saa.ars.usda.gov.

TABLE 1. Bacterial strains tested with the *C. perfringens* real-time PCR assay[a]

Isolate	Detection
Acinetobacter baumanii WT	−
Arcobacteria butzleri ATCC 49616	−
Bifidobacterium longum ATCC 15708	−
Bifidobacterium sp. strain 1-1-4 WT	−
Bifidobacterium sp. 2-1-1 strain WT	−
Brevundimonas diminuta WT	−
Campylobacter jejuni subsp. *jejuni* ATCC 29428	−
Campylobacter jejuni ATCC 33250	−
Campylobacter lari ATCC 35221	−
Campylobacter lari ATCC 43675	−
Clostridium absonum ATCC 27555	−
Clostridium acetobutylicum PI 657	−
Clostridium beijerinckii NRRL B-594	−
Clostridium novyi ATCC 19402	−
Clostridium novyi ATCC 27606	−
C. perfringens ATCC 12916	+
C. perfringens ATCC 13124	+
C. perfringens ATCC 3624	+
C. perfringens ATCC 3626	+
Clostridium rubrum PI 650	−
Clostridium sordelli ATCC 9714	−
Clostridium sporogenes ATCC 3584	−
Clostridium tetani PI 676	−
Clostridium tetanomorphum PI 677	−
Enterobacter cloacae ATCC 23355	−
E. coli ATCC 25922	−
Flavobacterium odoratum WT	−
Klebsiella pneumonia ATCC 13883	−
Lactobacillus acidophilus WT	−
Ochrobactrum sp. WT	−
Proteus vulgaris ATCC 13315	−
Pseudomonas aeruginosa ATCC 27853	−
Salmonella enteritidis WT	−
Salmonella typhimurium ATCC 14028	−
Serratia marcescens ATCC 8100	−
Sphingomonas paucimobilis WT	−
Staphylococcus aureus ATCC 25923	−
Staphylococcus epidermidis ATCC 12228	−
Staphylococcus gallinarum ATCC 35539	−
Streptococcus gordonii WT	−
Streptococcus pyogenes ATCC 19615	−

[a] WT, wild type. WT strains were isolated from chicken carcass rinses or chicken cecal material and identified by fatty acid analysis and/or biochemical tests. ATCC, American Type Culture Collection; PI, Presque Isle; NRRL, Northern Regional Research Laboratory.

(TAMRA) (5′-[FAM]TCATCATTCAACCAAAGGAGCAATCC[TAMRA]3′). The primers chosen yielded a 105-bp product. The 3′ end of the forward primer and a 5′ section of the hybridization probe overlap the forward primer used in a conventional PCR assay previously reported to be specific for *C. perfringens* (38).

Bacterial strains. *Clostridium* spp. and other bacteria used for specificity testing are listed in Table 1.

DNA extraction and real-time PCR. For cecal and ileal content samples, a Mo Bio (Solana Beach, CA) UltraClean Fecal DNA kit was employed to extract total DNA. The procedure was slightly modified from that provided by the manufacturer in the following ways: cecal or ileal intestinal contents were diluted 1:10 (wt/vol) with phosphate-buffered saline (pH 7.0) containing 0.05% Tween 20 (polyethylene glycol sorbitan monolaurate; Sigma-Aldrich, St. Louis, MO) and vortexed vigorously for 1 min. This dilution has been previously shown to be necessary for efficient bacterial lysis and DNA recovery in chicken gastrointestinal tract samples (4). One ml of the gastrointestinal (GI) tract slurry was placed in a bead tube, and the cells were lysed using a TurboMix apparatus (Scientific Industries, Bohemia, NY) attached to a vortexer and mixed at the highest setting for 10 min. A final volume of 50 μl was used to elute purified DNA from the spin column. For DNA extraction from bacterial pure cultures, a Mo Bio UltraClean Microbial Genomic DNA isolation kit was used by following the manufacturer's recommended procedure.

Taqman Universal PCR Master Mix (Applied Biosystems) was employed for the real-time PCR. Extensive optimization was performed with the primer/probe set on the following parameters: annealing temperature, primer concentration, probe concentration, primer ratios, and cycling conditions (two-step versus three-step). The assay was developed for use on a R.A.P.I.D. LightCycler instrument (Idaho Technology, Salt Lake City, UT). The standard reaction consisted of 10 μl of 2× Master Mix, 2 μl of bovine serum albumin (Idaho Technology) at 2.5 mg/ml (final concentration, 250 μg/ml), 1 μl of a 20 μM stock of primer CP165F (final concentration, 1 μM), 1 μl of primer CP269R at 20 μM (final concentration, 1 μM), and 1 μl of probe CP187F at 2 μM (final concentration, 0.1 μM). The template volume was 5 μl, yielding a final 20-μl volume in the R.A.P.I.D. capillary. The cycling conditions were as follows: an initial 10-min step at 94°C to activate the AmpliTaq Gold (Applied Biosystems) in the Master Mix, followed by 45 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 20 s, and extension at 70°C for 10 s. Fluorescence was acquired following the annealing step. The cycle threshold (C_t), or crossing point, was determined with the LightCycler data analysis software (Roche, Indianapolis, IN) included with the R.A.P.I.D. instrument, using the Fit Points method. Briefly, a threshold line was manually defined above the noninformative fluorescent data (noise band). A set of data points from the log-linear region of the fluorescent curves was then selected, and this set of points was used to generate a "best-fit" regression line, or crossing line. The intersection of the fluorescent curves with the crossing line determined the fractional cycle number of the crossing point.

Determination of specificities, limits of detection, and constructions of standard curves. Specificity testing of the assay was completed with approximately 1.5 ng of DNA purified from the strains listed in Table 1. The limit of detection for the assay with purified nucleic acid was performed with *C. perfringens* DNA (ATCC 13124) dilutions in duplicate and was repeated three times. To assay the sensitivities of whole cells, an overnight culture of *C. perfringens* ATCC 13124 was serially diluted and enumerated microscopically using a Petroff-Hausser counting chamber. The dilutions were then extracted with a Mo Bio UltraClean Fecal DNA kit following the aforementioned procedure to purify DNA and subjected to the real-time PCR procedure. The dilution series and extraction procedure was repeated three times. To create standard curves with known amounts of *C. perfringens* in gastrointestinal tract samples, samples of cecal and ileal material were acquired from a commercial chicken processing plant and from drug-free commercial broiler houses (see below). Samples that were *C. perfringens* negative by enrichment in modified iron-milk medium (5) and negative by the real-time PCR assay were spiked with known quantities of *C. perfringens* enumerated via standard plate counts. To determine assay variation as a result of samples from birds of different ages, the spiking series was repeated on four occasions, twice with cecal and ileal samples from 6- to 7-week-old birds and twice with cecal and ileal samples from 3-week-old birds. The spiked samples were extracted with a Mo Bio UltraClean Fecal kit in the fashion described above, and the purified total DNA was used to construct standard curves for subsequent quantification of unknowns.

Cecal PCR inhibition. Total DNA was purified using a Mo Bio UltraClean Fecal DNA kit (as described above) from a cecal sample obtained from an approximately 3-week-old bird that was negative for *C. perfringens* by direct plating and enrichment culture. The DNA, which was also negative for *C. perfringens* by the real-time assay, was then combined in various quantities to known amounts of *C. perfringens* (ATCC 13124) genomic DNA, and the combination was used as template for the real-time PCR assay.

Detection, enrichment, and quantification of *C. perfringens* in the gastrointestinal tract of commercially reared broiler chickens. To assess the ability of the real-time PCR assay to measure amounts of *C. perfringens* in broilers, gastrointestinal tract samples were acquired from three separate commercial chicken houses in northeastern Georgia, which had each recently experienced a necrotic enteritis outbreak. These facilities originally housed birds reared without the addition of growth-promoting antibiotics, but were being treated therapeutically with antibiotics at the time of the sampling. From the first farm, which was sampled on 26 May 2004, five seemingly healthy birds were sacrificed by cervical dislocation and dissected at the premises, and the GI tracts were removed and placed on ice for transport to the laboratory. Five moribund or recently dead (<6 h) birds were also dissected and their GI tracts obtained. From each of the next two farms, sampled on 6 July 2004 and 15 July 2004, GU tracts from 10 apparently healthy and 10 affected birds were collected in the same manner. Luminal material from the cecum and ileum (approximately 18 to 24 cm above the cloaca) was removed in the laboratory, weighed, diluted 10-fold, and subjected to the DNA purification and real-time PCR procedure described above. Concurrently, *C. perfringens* direct counts were performed with dilutions spread on tryptose-sulfite-cycloserine agar supplemented with 50% egg yolk emulsion (5). Also, *C. perfringens* enrichments were performed by adding 1 ml of the above mixture to

modified iron-milk medium (5) and incubating overnight. Presumptive positive tubes (stormy fermentation) were then confirmed by subculture on tryptose-sulfite-cycloserine agar and by subjecting typical *C. perfringens* colonies (i.e., those with lecithinase activity and sulfide production) to the real-time PCR procedure.

Diagnostic specificity and sensitivity were calculated by comparing the qualitative results of *C. perfringens* isolation in culture (yes/no) to qualitative direct real-time PCR results (detected/not detected). Diagnostic specificity was calculated as the number of samples that were negative by both real-time PCR and by culture divided by the sum of this number and the number of samples that were positive by real-time PCR but negative by culture. Diagnostic sensitivity was calculated as the number of samples that were positive by both real-time PCR and culture divided by the sum of this number and the number of samples that were negative by real-time PCR but positive by culture.

For quantitative comparisons, the results of the direct plate counts were compared to the real-time PCR results obtained using the standard curves constructed with the appropriate sample type. The base 10 logarithm of the plate count was plotted versus the base 10 logarithm of the real-time PCR quantitative estimate, and "best-fit" linear regression lines were drawn through the origin. Samples that were below the limit of detection by either culturing or real-time PCR were excluded from the analysis, as were those too numerous to accurately quantify via the standard plate count procedure.

RESULTS

Specificity. The specificity of the assay was tested against a panel of genomic DNA preparations (Table 1) that included a number of wild-type strains isolated from chicken cecal droppings and carcass rinses. The primer/probe set detected only strains classified as *C. perfringens*, and no signal was detected for any other species, including the close relatives in cluster I (12) of the *Clostridium* genus.

Sensitivity and variability. The sensitivity of the assay was evaluated with purified *C. perfringens* (ATCC 13124 DNA) genomic DNA, whole *C. perfringens* cells, and *C. perfringens*-negative cecal and ileal samples spiked with *C. perfringens* cells. Figure 1 illustrates the logarithmic-linear plot of the cycle threshold values versus the concentration of DNA or cell number. With pure DNA, the reproducible limit of detection was approximately 50 fg of *C. perfringens* genomic DNA (Fig. 1A). With whole cells, the assay could consistently detect approximately 20 cells (Fig. 1B). To obtain information on interassay variation of the real-time PCR as a result of different sample types, a spiking series was performed four times on four different days with two cecal and two ileal samples obtained from 3-week-old birds and two cecal and two ileal samples from 6- to 7-week-old birds. The samples were originally confirmed to be negative by direct plating, selective enrichment, and real-time PCR. They were then spiked with enumerated *C. perfringens*, and DNA was extracted in the standard fashion. As illustrated in Table 2, with the spiked ileal samples from the 6- to 7-week-old birds, the assay could reproducibly detect approximately 10^2 CFU/g of ileal material; however, with spiked cecal samples from the 6- to 7-week-old birds, the consistent limit of detection was only about 10^4 CFU/g of cecal material. With GI tract material from the 3-week-old broilers, the reproducible detection limits with spiked cecal and ileal samples were 10^3 and 10^2 CFU/g, respectively. The coefficient of variation (CV) for the four spiked series ranged from 1.44 to 2.73% for the cecal samples and from 1.65 to 4.72% with the samples from the ileum.

Inhibition. The low limit of detection observed with spiked ileal samples compared to spiked cecal samples suggested that the DNA extraction procedure did not remove all PCR inhibitors from the cecal material. To detect the effect of inhibitors, DNA was extracted from a chicken cecal sample that was previously shown to be negative for *C. perfringens* by direct and enrichment culture. The cecal DNA, also negative with the real-time PCR assay, was then added in various amounts to purified *C. perfringens* genomic DNA to assess its effect on the PCR results. As illustrated in Table 3, the results suggested that the effect of the cecal inhibitor was dependent on the initial target concentration. When 230 pg or 23 pg of genomic *C. perfringens* DNA (each in a 1-μl volume) was used as template in the reaction, the addition of between 1 and 4 μl of the cecal DNA extraction did not affect C_t. However, with 2.3 pg of genomic *C. perfringens*, the addition of 1 μl of inhibitor increased the C_t from 36.08 to 43.26, and with 2 μl of the inhibitor, the assay could not detect the *C. perfringens* DNA. With 230 fg of *C. perfringens* DNA as template, 1 μl of inhibitor was sufficient to prevent nucleic acid detection.

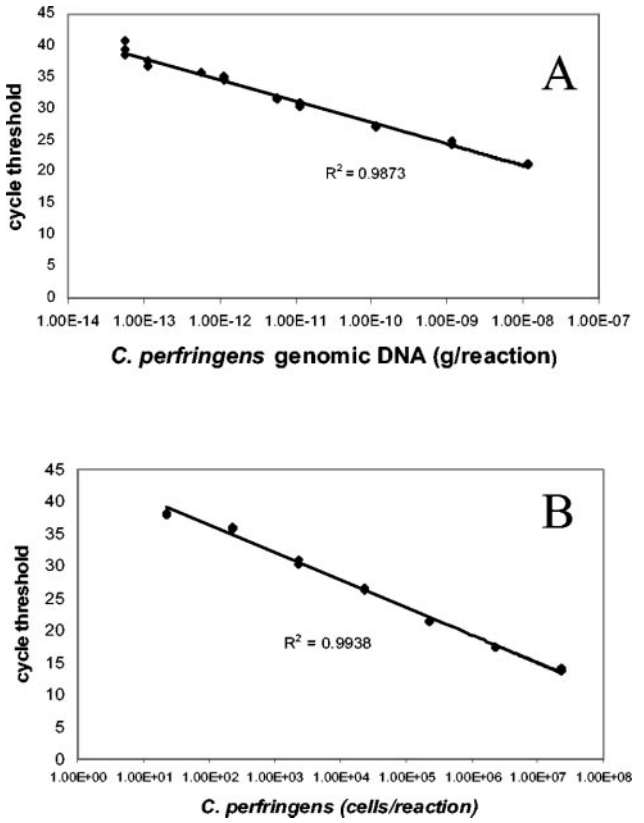

FIG. 1. Logarithmic-linear plots of the *C. perfringens* real-time PCR assay amplification profile for diluted purified genomic DNA (A) and *C. perfringens* whole cells (B). The amounts of DNA or numbers of cells are plotted versus the cycle threshold values. Experiments with three replicate samples were performed for each dilution.

Diagnostic specificity and sensitivity. One hundred chicken GI tract samples, 50 from the ileum and 50 from the cecum, obtained from drug-free broiler operations were screened for *C. perfringens* using the real-time PCR assay. Concurrently, the samples were tested for the occurrence of *C. perfringens* by direct culturing on selective medium and by overnight nonselective enrichment followed by plating on the selective medium. The results are presented in Table 4. With both sample

TABLE 2. Interassay variability among four spiked samples from broiler gastrointestinal tracts[a]

CFU	C_t for indicated sample no. (broiler age)								CV (%) for:	
	1 (6–7 wk old)		2 (3 wk old)		3 (6–7 wk old)		4 (3 wk old)		Ceca	Ilea
	Cecum	Ileum	Cecum	Ileum	Cecum	Ileum	Cecum	Ileum		
1.00E+00	ND	ND	ND	ND	ND	ND	ND	ND		
1.00E+01	ND	ND	ND	ND	ND	41.29	ND	40.08		
1.00E+02	ND	39.44	33.35	37.78	ND	38.20	ND	35.85		3.41
1.00E+03	ND	35.04	30.17	34.06	ND	35.11	33.67	32.88		2.64
1.00E+04	30.82	33.47	28.61	31.21	30.33	33.10	29.9	30.92	2.73	3.49
1.00E+05	24.90	29.08	25.25	26.08	24.69	29.12	25.64	27.04	1.44	4.72
1.00E+06	21.41	25.02	21.29	23.80	21.13	25.14	21.99	22.68	1.51	4.15
1.00E+07	17.73	20.98	18.02	21.13	17.25	21.34	18.51	20.41	2.56	1.65

[a] The coeffiecient of variation was calculated only when all four replicates were detected. ND, not detected.

types combined, the diagnostic specificity for comparison of real-time PCR to culture was 0.77, and the diagnostic sensitivity was 0.91. When only cecal samples were considered, the diagnostic specificity increased to 0.95, but the diagnostic sensitivity was 0.83. An examination of just the ileal samples resulted in a diagnostic specificity of only 0.53 but a diagnostic sensitivity of 0.97. The real-time PCR assay is likely more sensitive than culture for many ileal samples, hence the low calculated diagnostic specificity.

Quantification of *C. perfringens* in chicken gastrointestinal samples. To estimate the levels of *C. perfringens* in the GI tract by quantitative PCR, the observed C_t values obtained with 47 of the unknown samples were plotted on the standard curves made with spiked cecal material or spiked ileal material, as appropriate. The log of the quantitative real-time PCR estimates was then compared to the log of the viable plate counts for each unknown sample, and the results are illustrated in Fig. 2. With both sample types considered together, the best-fit regression line drawn through the origin revealed a value of 0.79 for the square of the correlation coefficient (r^2). With just the cecal samples, r^2 was 0.37, but with the ileal samples, the scatter was much reduced, with an r^2 value of 0.95.

TABLE 3. Effect of cecal inhibitor on detection of *C. perfringens* with the real-time PCR assay

C. perfringens DNA (pg)	Potential inhibitor (μl)[a]	Cycle threshold
230	4	27.24
	2	26.32
	1	26.98
	0	27.16
23	4	30.96
	2	31
	1	30.49
	0	31.18
2.3	4	ND[b]
	2	ND
	1	43.26
	0	36.08
0.23	4	ND
	2	ND
	1	ND
	0	40.83

[a] DNA extraction from a cecal sample that was negative by PCR and by direct and enrichment culture.
[b] ND, not detected.

DISCUSSION

Herein, a simple and rapid culture-independent real-time PCR assay was described for quantification of *C. perfringens* in avian intestinal samples. To date, there has been only limited use of real-time PCR to detect or quantify this species. The 16S rDNA primer pair designed by Wang et al. (38) has been employed in a method using the nonspecific DNA binding dye SYBR green to estimate *C. perfringens* DNA relative to total bacterial DNA in mucosal samples from piglets (14) and mucosal and luminal samples from chickens (11). Fukushima et al. (17) used primers directed toward the *C. perfringens* enterotoxin gene as part of a suite of real-time SYBR green assays for the detection of food- and waterborne pathogens in feces. To our knowledge, no *C. perfringens* real-time methods that use hydrolysis probes, fluorescence resonance energy transfer probes, hairpin probes (i.e., molecular beacons) or any other reporter probe designed to hybridize to the PCR amplicon have been developed. The probe provides for another level of specificity beyond the primer pair, increasing confidence that only the target sequence, and no spurious products or primer-dimers, will be detected.

PCR from complex substrates, such as feces, is known to be problematic due to the presence of inhibitors (40), such as polyphenolic compounds (25); gastrointestinal tract samples are expected to suffer from the same limitation. Although the DNA extraction and purification procedure employed here provides a step to specifically remove inhibitors, when target DNA purified from cecal contents was present at low copy numbers, the sensitivity of the assay was still shown to be

TABLE 4. Diagnostic sensitivity and specificity of the *C. perfringens* real-time PCR assay

Real-time PCR result	Culture result for:								
	Cecal and ileal samples			Cecal samples only			Ileal samples only		
	+	−	Total	+	−	Total	+	−	Total
+	59	8	67	25	1	26	34	7	41
−	6	27	33	5	19	24	1	8	9
Total	65	35	100	30	20	50	35	15	50

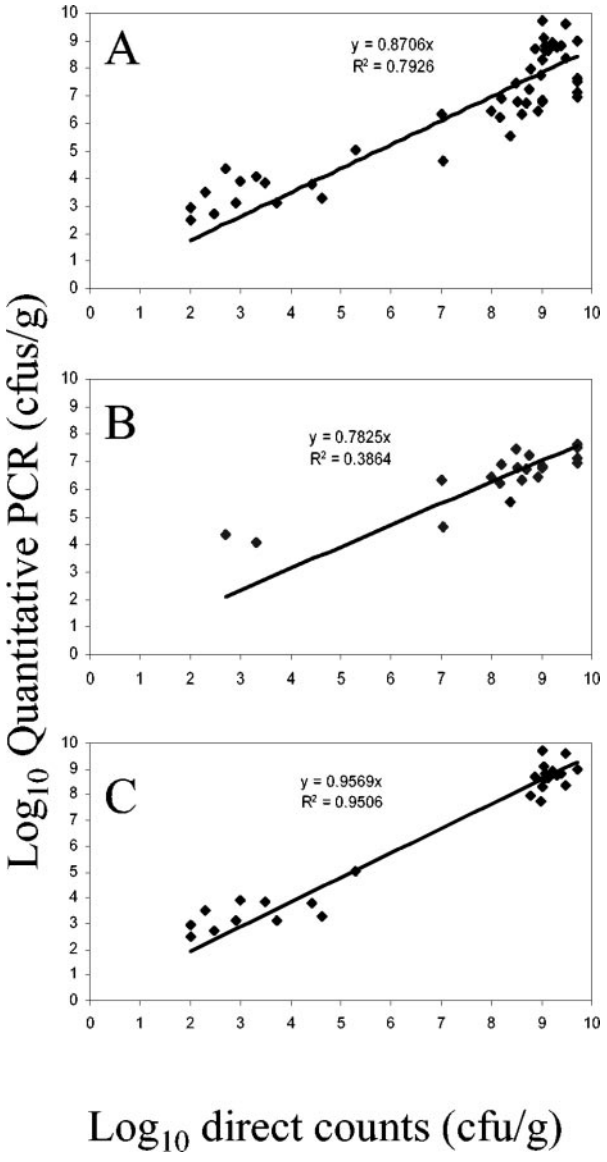

FIG. 2. Quantification comparison of *C. perfringens* estimates for gastrointestinal tract samples taken from commercial broilers by direct plate counts and quantitative real-time PCR. Shown are results for both cecal and ileal samples (A), cecal samples only (B), and ileal samples only (C).

adversely affected by chemical inhibitors. Inhibition is much less of an issue with ileal material, as the assay with spiked ileal samples was two orders of magnitude more sensitive than that with spiked cecal samples. The reduced amount of inhibitor presumably accounts for the increased diagnostic sensitivity observed with these samples. Furthermore, the positive correlation between culturable plate counts and quantitative real-time PCR estimates was much stronger with DNA extracted from ileal material than from cecal material. Certainly the discrepancy between the quantitative methods compared here, i.e., culture of viable organisms and detection of nucleic acid via PCR, could be due to the detection of dead cells or noncellular DNA by PCR. However, despite the inherent differences in methodology, quantitative real-time PCR correlates well with viable counts, suggesting that for research purposes, quantitative real-time PCR can supplant the need for traditional plate counts, especially for samples from the ileum. With regard to the study of necrotic enteritis and dysbacteriosis, this is fortuitous, since necrotic enteritis is a phenomenon initiated largely in the middle gut region of the fowl gastrointestinal tract.

The limit of detection of the assay with DNA extracts from cecal samples, although less sensitive than that for the ileal samples, was nevertheless in the general range of those of other recent real-time PCR methods designed to detect intestinal bacteria. For example, a quantitative real-time assay targeted toward *Bifidobacterium* spp. reported a limit of detection at 5×10^4 cells/g feces (18), and Belanger et al. (9) found that their real-time PCR test for *Clostridium difficile* in feces had a limit of detection of 5×10^4 CFU/g feces. A conventional multiplex PCR assay targeted to the *C. perfringens* alpha and enterotoxin genes used to detect the bacterium in pig feces and intestinal contents was reported to have an average sensitivity of 9.2×10^4 CFU/g (22). We choose to focus on the 16S rRNA gene for development of this assay, since visual examination of multiple sequence alignments revealed a short region unique to *C. perfringens*, which was previously exploited by Wang et al. (38). Also, this bacterium has 10 rRNA operons (36), and although there are small numbers of heterologous base positions among the 16S rRNA gene copies in this species, the assay should have increased sensitivity versus a target with one or two gene copies.

Assessments of the natural bacterial populations that comprise the gastrointestinal tract of chickens, whether by traditional culturing (6, 7, 30, 35) or by molecular methods (26, 41), have suggested a complex community dominated by gram-positive bacteria. By both methods, *C. perfringens* has been identified as a common member of this community (26, 37). However, the occurrence of necrotic enteritis is sporadic, and the factors that affect the onset of the disease state are not well understood. High levels of *C. perfringens* are associated with necrotic intestinal tissue, and birds from healthy flocks are reported to have much lower culturable numbers of cells (13). The utility of this assay will perhaps be best realized in the study of the gut microbial population dynamics of poultry reared under drug-free conditions. In these systems, the microbial community in the ileum is documented to be more diverse than that for birds raised with antibiotic growth promoters (24), and such birds are more vulnerable to diseases of bacterial imbalance, such as necrotic enteritis (8).

Additionally, the ability to rapidly detect and quantify *C. perfringens* in complex samples would be useful in circumstances beyond the avian samples described here. For example, *C. perfringens* is a widely distributed and frequent cause of food-borne disease often associated with food service modes of distribution and delivery (29). While the 16S rRNA species-specific test described here was not tested on food matrices, the potential exists for it to be used in combination with the epidemiologically relevant *C. perfringens* enterotoxin (CPE) marker assay (39) for chromosomally encoded CPE. Expedited detection and quantification of CPE-positive strains relative to the total *C. perfringens* population may prove useful for screening at-risk food products or for use in outbreak situations.

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REFERENCES

1. **Al-Sheikhly, F., and R. B. Truscott.** 1977. The interaction of *Clostridium perfringens* and its toxins in the production of necrotic enteritis of chickens. Avian Dis. **21:**256–263.
2. **Al-Sheikhly, F., and R. B. Truscott.** 1977. The pathology of necrotic enteritis of chickens following infusion of broth cultures of *Clostridium perfringens* into the duodenum. Avian Dis. **21:**230–240.
3. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
4. **Apajalahti, J. H., L. K. Sarkilahti, B. R. Maki, J. P. Heikkinen, P. H. Nurminen, and W. E. Holben.** 1998. Effective recovery of bacterial DNA and percent-guanine-plus-cytosine-based analysis of community structure in the gastrointestinal tract of broiler chickens. Appl. Environ. Microbiol. **64:**4084–4088.
5. **Atlas, R. M.** 1997. Handbook of microbiological media. CRC Press, Boca Raton, Fla.
6. **Barnes, E. M.** 1979. The intestinal microflora of poultry and game birds during life and after storage. J. Appl. Bacteriol. **46:**407–419.
7. **Barnes, E. M., G. C. Mead, D. A. Barnum, and E. G. Harry.** 1972. The intestinal flora of the chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. Br. Poult. Sci. **13:**311–326.
8. **Bedford, M.** 2000. Removal of antibiotic growth promoters from poultry diets: implications and strategies to minimise subsequent problems. World's Poult. Sci. J. **56:**347–365.
9. **Belanger, S. D., M. Boissinot, N. Clairoux, F. J. Picard, and M. G. Bergeron.** 2003. Rapid detection of *Clostridium difficile* in feces by real-time PCR. J. Clin. Microbiol. **41:**730–734.
10. **Cockerill, F. R., and J. R. Uhl.** 2002. Applications and challenges of real-time PCR for the clinical microbiology laboratory, p. 3–27. *In* U. Reischl, C. Wittwer, and F. Cockerill (ed.), Rapid cycle real-time PCR: methods and applications. Springer Verlag, Berlin, Germany.
11. **Collier, C. T., J. D. van der Klis, B. Deplancke, D. B. Anderson, and H. R. Gaskins.** 2003. Effects of tylosin on bacterial mucolysis, *Clostridium perfringens* colonization, and intestinal barrier function in a chick model of necrotic enteritis. Antimicrob. Agents Chemother. **47:**3311–3317.
12. **Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. Farrow.** 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. **44:**812–826.
13. **Craven, S. E.** 2000. Colonization of the intestinal tract by *Clostridium perfringens* and fecal shedding in diet-stressed and unstressed broiler chickens. Poult. Sci. **79:**843–849.
14. **Deplancke, B., O. Vidal, D. Ganessunker, S. M. Donovan, R. I. Mackie, and H. R. Gaskins.** 2002. Selective growth of mucolytic bacteria including *Clostridium perfringens* in a neonatal piglet model of total parenteral nutrition. Am. J. Clin. Nutr. **76:**1117–1125.
15. **Elwinger, K., C. Schneitz, E. Berndtson, O. Fossum, B. Teglof, and B. Engstom.** 1992. Factors affecting the incidence of necrotic enteritis, caecal carriage of *Clostridium perfringens* and bird performance in broiler chicks. Acta Vet. Scand. **33:**369–378.
16. **Finken, M. D., and D. P. Wages** 1997. Necrotic enteritis, p. 261–267. *In* B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald, and Y. M. Said (ed.), Diseases of poultry. Iowa State University Press, Ames, Iowa.
17. **Fukushima, H., Y. Tsunomori, and R. Seki.** 2003. Duplex real-time SYBR green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. J. Clin. Microbiol. **41:**5134–5146.
18. **Gueimonde, M., S. Tolkko, T. Korpimaki, and S. Salminen.** 2004. New real-time quantitative PCR procedure for quantification of bifidobacteria in human fecal samples. Appl. Environ. Microbiol. **70:**4165–4169.
19. **Hatheway, C. L.** 1990. Toxigenic clostridia. Clin. Microbiol. Rev. **3:**66–98.
20. **Kaldhusdal, M.** 1999. Necrotic enteritis as affected by dietary ingredients. Proceedings 12th European Symposium on Poultry Nutrition, p. 301–310. World's Poultry Science Association, Veldhoven, The Netherlands.
21. **Kaldhusdal, M., and M. Hofshagen.** 1992. Barley inclusion and avoparcin supplementation in broiler diets. 2. Clinical, pathological, and bacteriological findings in a mild form of necrotic enteritis. Poult. Sci. **71:**1145–1153.
22. **Kanakaraj, R., D. L. Harris, J. G. Songer, and B. Bosworth.** 1998. Multiplex PCR assay for detection of *Clostridium perfringens* in feces and intestinal contents of pigs and in swine feed. Vet. Microbiol. **63:**29–38.
23. **Kato, N., S. M. Kim, H. Kato, K. Tanaka, K. Watanabe, K. Ueno, and Y. Chong.** 1993. Identification of enterotoxin-producing *Clostridium perfringens* by the polymerase chain reaction. Jpn. J. Infect. Dis. **67:**724–729.
24. **Knarreborg, A., M. A. Simon, R. M. Engberg, B. B. Jensen, and G. W. Tannock.** 2002. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. Appl. Environ. Microbiol. **68:**5918–5924.
25. **Koonjul, P. K., W. F. Brandt, J. M. Farrant, and G. G. Lindsey.** 1999. Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA. Nucleic Acids Res. **27:**915–916.
26. **Lu, J., U. Idris, B. Harmon, C. Hofacre, J. J. Maurer, and M. D. Lee.** 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Appl. Environ. Microbiol. **69:**6816–6824.
27. **Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Parker, Jr., P. R. Saxman, R. J. Farris, G. M. Garrity, G. J. Olsen, T. M. Schmidt, and J. M. Tiedje.** 2001. The RDP-II (Ribosomal Database Project). Nucleic Acids Res. **29:**173–174.
28. **Mateos, G. G., R. Lazaro, and M. I. Gracia.** 2002. The feasibility of using nutritional modifications to replace drugs in poultry feed. J. Appl. Poult. Res. **11:**437–452.
29. **McClane, B. A.** 1997. *Clostridium perfringens*, p. 305–326. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers. ASM Press, Washington, D.C.
30. **Mead, G. C., and B. W. Adams.** 1975. Some observations on the caecal microflora of the chick during the first two weeks of life. Br. Poult. Sci. **16:**169–176.
31. **Parish, W. E.** 1961. Necrotic enteritis in the fowl. II. Examination of the causal *Clostridium welchii*. J. Comp. Pathol. **71:**394–404.
32. **Prescott, J. F., R. Sivendra, and D. A. Barnum.** 1978. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. Can. Vet. J. **19:**181–183.
33. **Rood, J. I., and S. T. Cole.** 1991. Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiol. Rev. **55:**621–648.
34. **Rozen, S., and H. J. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers, p. 365–386. *In* S. Misener and S. A. Krawetz (ed.), Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, N.J.
35. **Salanitro, J. P., I. G. Fairchilds, and Y. D. Zgornicki.** 1974. Isolation, culture characteristics, and identification of anaerobic bacteria from the chicken cecum. Appl. Microbiol. **27:**678–687.
36. **Shimizu, T., S. Ohshima, K. Ohtani, T. Shimizu, and H. Hayashi.** 2001. Genomic map of *Clostridium perfringens* strain 13. Microbiol. Immunol. **45:**179–189.
37. **Tschirdewahn, B., S. Notermans, K. Wernars, and F. Untermann.** 1991. The presence of enterotoxigenic *Clostridium perfringens* strains in faeces of various animals. Int. J. Food Microbiol. **14:**175–178.
38. **Wang, R. F., W. W. Cao, W. Franklin, W. Campbell, and C. E. Cerniglia.** 1994. A 16S rDNA-based PCR method for rapid and specific detection of *Clostridium perfringens* in food. Mol. Cell. Probes **8:**131–137.
39. **Wen, Q., K. Miyamoto, and B. A. McClane.** 2003. Development of a duplex PCR genotyping assay for distinguishing *Clostridium perfringens* type A isolates carrying chromosomal enterotoxin (*cpe*) genes from those carrying plasmid-borne enterotoxin (*cpe*) genes. J. Clin. Microbiol. **41:**1494–1498.
40. **Wilde, J., J. Eiden, and R. Yolken.** 1990. Removal of inhibitory substances from human fecal specimens for detection of group A rotaviruses by reverse transcriptase and polymerase chain reactions. J. Clin. Microbiol. **28:**1300–1307.
41. **Zhu, X. Y., T. Zhong, Y. Pandya, and R. D. Joerger.** 2002. 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Appl. Environ. Microbiol. **68:**124–137.